Abstract

Obesity is a worldwide problem of public health which is taking epidemic proportions. One of the main consequences of obesity is the development of cardiovascular diseases, which in turn, is the main cause of death in Brazil and in the world.

**Aim:** To evaluate functional and morphological changes in the mesenteric artery, kidney and liver in obesity induced by high fat diet plus fructose.

**Methods:** Male Wistar rats were submitted to a normolipidic diet (3.8% of fat–control group) or hyperlipidic diet (59% fat–HFD+F group) associated with fructose (100 mg/ml) in the drinking water during 12 weeks, starting at the 4th week of life. Initial and final body weight and epididymal fat, glucose tolerance and lipidic profile were evaluated. The superior mesenteric artery was removed for functional and histological analysis. Renal and hepatic functions were measured by plasma levels of specific metabolites and enzymes. The kidney and liver were also collected for histology.

**Results:** In HFD+F group, it was observed increased body weight gain, epididymal fat and plasma levels of triglycerides while glucose tolerance was diminished. In the mesenteric artery, endothelium-dependent relaxation was reduced, with no changes in the endothelium-independent relaxation. Morphologically, no changes were seen in the vascular endothelium and smooth muscle. The kidney did not present functional and histological changes whereas the liver presented lipid accumulation, without changes on its function.

**Conclusion:** Our data suggest that the high fat diet plus fructose induced endothelial dysfunction without structural changes on the vascular endothelium and smooth muscle. In this model of obesity, renal function and morphology were preserved while the hepatic tissue showed histological changes which are suggestive of a simple non-alcoholic steatosis.

**Keywords:** Obesity; High fat diet; Fructose; Mesenteric artery; Kidney; Liver

Introduction

Obesity has been described as an important public health problem and is gaining prominence in the global epidemiological scenario. Its prevalence has increased in recent decades all over the world, including developing countries, like Brazil, where problems of malnutrition used to prevail [1]. Characterized by excessive accumulation of adipose tissue located throughout the body, obesity is a chronic disease that often causes damage to health. This increase can be caused by multiple factors related to excessive intake of high fat foods, reduction of physical exercises, genetics and metabolic, social, behavioral and cultural factors [2].

The local control of the vascular tone is regulated by the equilibrium of contracting and relaxing mediators released from the endothelial cells. Endothelial dysfunction refers to an imbalance in endothelial production of these mediators and might be also referred to a decrease in endothelium-dependent relaxation caused by decreased nitric oxide (NO) bioavailability, although the production of other endothelium-derived vasoactive substances such as PG12, EDHF, ET-1, Ang II, TXA2, may also be altered [3]. NO is one of endothelium-derived relaxing factors of greatest importance, directly related to the integrity of endothelial function. NO also have several antithrombic properties which include inhibition of monocyte, leukocyte and platelet adhesion, antioxidant and inhibition of muscle cell proliferation. The reduced bioavailability of NO seems to be present in cardiovascular disease [4]. Studies have shown that vasodilation mediated by the endothelium is initially reduced in the process of atherosclerosis, even before angiographic morphological changes. This vasodilation progressively decreases as the severity of atherosclerosis increases and also due to risk factors such as hypertension, dyslipidemia and physical inactivity [4]. Endothelial dysfunction is presented in various metabolic and/or cardiovascular disorders, such as obesity, glucose intolerance, hyperglycemia (diabetes mellitus), hypertension and dyslipidemia. In all these conditions insulin resistance, which represents itself a metabolic disorder manifested by a reduction in the use of glucose by peripheral skeletal muscle, has been strongly associated with early endothelial dysfunction [3].

Additionally, it has been shown that obesity is a potential risk factor for developing kidney and liver disease. Glomerulopathy seems to be the main kidney disease related to obesity [5,6]. Similarly,
hepatic steatosis is also known to be a consequence of high fat diet consumption. In addition, increased insulin levels inhibit the hepatic production of glucose and induce lipogenesis [7].

Hence, the aim of this work was to evaluate the impact of a high fat diet associated with fructose consumption in the function and morphology of the mesenteric artery, kidney and liver of rats.

Materials and Methods

Animals and experimental protocols

Four weeks old Wistar rats from CEMIB-UNICAMP, were housed in collective cages (5 rats per cage) and kept in a light/dark cycle (12/12 hours), receiving water and chow ad libitum. The experimental protocols were approved by the Ethics and Research Committee of the Universidade São Francisco (protocol number 004.06.11).

Induction of obesity by a high-fat diet plus fructose overload

Obesity was induced by association of a high fat diet and an overload with fructose (100 mg/ml- Lab Synth, Diadema–SP, Brazil). Fructose was added in the tap water of rats receiving high fat diet only. The high fat diet was commercially purchased (Prag solutions Biosciences, Jaú, SP) and provided 5.5 kcal/g (being 59% lipids, 18% proteins and 23% carbohydrates). Standard chow for rodents provided 3 kcal/g (being 40% carbohydrates, 3.8% lipids and 26.5% proteins). The high fat diet and fructose were given during 12 weeks, beginning after on the weaning at the 4th week of life.

Body weight was monitored weekly. At the end of the study, we conducted the evaluation of the total body weight of the animals and after sacrifice the epididymal fat was isolated and weighed for analysis of the accumulation of adipose tissue of both groups.

Glucose tolerance test and lipid profile

The glucose tolerance test was performed after 14 hours of fasting. During the fasting period, the animals had free access of water, without fructose addition.

For the glucose tolerance test, capillary blood was collected from the tail artery of the rats. Blood samples were collected at the times 0, 10, 20, 30, 40 and 60 minutes after dextrose overload (2 g/Kg). Glucose concentrations were measured using a glucometer (ONE TOUCH ULTRA, Accu Check Roche Diagnostics, Indianapolis, IN, USA).

To assess the lipid profile, the arterial blood was collected from the descending branch of the aorta. Serum was obtained by centrifugation (1,000×g, 10 minutes) and levels of total cholesterol (TC) and triglycerides (TG) were measured by enzymatic method using specific kits (LABTEST, Lagoa Santa, MG, Brazil).

Evaluation of hepatic and renal function

The serum and plasma samples were obtained as described in the previous section to evaluate the lipid profile.

Liver function was assessed by measurement of serum ALT and albumin, using specific kits (LABTEST, Lagoa Santa, MG, Brazil).

Renal function was assessed by measuring plasma levels of creatinine and urea, also through specific kits (LABTEST, Lagoa Santa, MG, Brazil).

Functional assessment of superior mesenteric artery

After the blood was collected, the superior mesenteric artery was removed and placed in chilled Krebs-Henseleit buffer with the following composition (mmol/L): NaCl, 130; NaHCO 3, 14.9; dextrose, 5.5; KCl, 4.7; KH 2PO 4, 1.18; MgSO 4·7H 2O, 1.17 and CaCl 2·2H 2O, 1.6. Tissues were cleaned from connective and adipose tissues and cut in rings (1 mm length) and mounted in an organ bath coupled to an isometric force transducer (Ugo Basili, Varese, Italy) connected to a Power Lab 8/SP™ data acquisition system (software Chart 5.0, AD Instruments, Colorado Springs, USA). The bathing solution was maintained at 37°C and continuously aerated with 95% O 2 and 5% CO 2. Tissues were allowed to equilibrate for 45 min under a resting tension of 10 mN. Rings were contracted with phenylephrine (PE, 10 μmol/L) and endothelial function was assessed applying acetylcholine (ACH, 1 μmol/L). Cumulative concentration-response curves to ACh (0.001 - 10 μmol/L) and the relaxation to one single dose of sodium nitroprusside (SNP, 1 μmol/L) were obtained in PE-contracted rings to evaluate endothelium-dependent and independent relaxation, respectively. Relaxation was evaluated only in rings that reached a plateau after PE-induced contraction. Vascular relaxation is expressed as percentage of PE-induced maximum contraction. Potency is given by the pEC 50 , which represents the concentration of each drug necessary to cause 50% of maximum relaxation and it is expressed as −log molar (mol/L).

Morphological evaluation of mesenteric artery, kidney and liver

Rings of mesenteric artery, kidney and liver fragments from the animals were collected and fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4), overnight, and then dehydrated in an ascending series of ethanol, 50% (1 h), 70% (overnight), 80%, 95% and 100% (30 min each). Then the material was diaphanized in ethanol: xylene (30 min), xylene (3×30 min, each) and infiltrated in xylene: wax (30 min.), pure paraffin (1 h+2 h) for inclusion.

Histological sections (5 μm) obtained were deparaffinized, rehydrated and stained with hematoxylin for 5 min. and eosin for 3 min (HE). The slides were washed in tap water and dehydrated in an ascending series of ethanol, mounted with Canada balsam synthetic and analyzed in an optical microscope Olympus BX51-PH-III (Olympus Optical Co., Tokyo, Japan).

After the in vitro experiments the arteries were fixed in 4% paraformaldehyde for histology in paraffin. Part of histological slides was stained with HE, for analysis of the general morphology of the mesenteric arteries, and part stained with Orcein (30 min) for analysis of elastic fibers.

The remaining slides were incubated with specific antibodies: eNOS and p47 phox (1:50), Ec-SOD and Mn-SOD (1:100) (Santa Cruz, Dallas, TX, USA). After being deparaffinized, the slides were submitted to blockade of endogenous peroxidase (30 min) and endogenous protein (PBS/1% BSA-60 min). The sections were exposed to primary antibody overnight, washed and exposed to secondary antibody incubation (2 h) using Dako Advance kit.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) for 4-5 experiments. Analyzes of variance were performed (two-way ANOVA) to determine differences between groups and Tukey post-test, using the program Graph Pad Prism 3.0. A significance level of p<0.05 was adopted.
Results

Initial and final body weight

Prior to the high fat diet administration, the animals were randomly assigned to the control group and HFD+F group and the basal body weight was evaluated. The body weight at the beginning was 113 ± 6 g for the control group and 110 ± 6 g for the HFD+F group. After the 12th week of study, the body weight of the control group was 414 ± 15 g while in the HFD+F group the final body weight was 504 ± 17 g (Figure 1A).

Epididymal fat

In animal models of obesity, visceral fat content has been correlated with abdominal fat in humans. After sacrifice, the epididymal fat was removed and weighed as an index of body fat percentage. It was observed that the HFD+F produced a significant increase in epididymal fat (18.76 ± 2.11 g for the HFD+F group), when compared to the control group (6.79 ± 1.20 g) (Figure 1B).

Glucose tolerance test (GTT)

Fasting glucose was similar between groups (78 ± 17 mg/dl and 92 ± 6 for control and HFD+F groups, respectively). After dextrose overload, glucose was measured after 10, 20, 30, 40 and 60 minutes of ingestion. The profile of blood glucose curve was not different between the groups at 10, 20, 30 and 40 minutes. However, after 60 minutes of overload, blood glucose was significantly higher in the HFD+F group compared to the control group (Figure 1C).

Lipid profile

After 12 weeks of treatment, there was no change in total cholesterol (45 ± 4 and 39 ± 4 mg/dl for control and HFD+F group, respectively). However, there was a significant increase in triglycerides of the HFD+F group (81 ± 6 mg/dl) compared to the control group (55 ± 6 mg/dl); (Figure 2).

Kidney function and morphology

Plasma levels of creatinine and urea were measured as an index of renal function to evaluate the effect of HFD+F on the kidneys. No changes were observed in plasma levels of creatinine and urea (Figure 3A and 3B). In addition to the renal function, the histology of corpuscle and renal glomeruli was assessed. HFD+F produced no structural changes in renal tissue (Figure 3C and 3D).

Liver function and morphology

Serum levels of ALT and albumin were evaluated as an index for hepatic function. After 12 weeks of HFD+F it was not observed changes in the ALT and albumin levels (Figure 4A and 4B). On the other hand, it was observed lipid accumulation in the liver of HFD+F group (Figure 4C and 4D).

Vascular reactivity and morphological evaluation of the mesenteric artery

Vascular reactivity was assessed in the mesenteric arteries from control and HFD+F groups. Concentration-response curves to acetylcholine were obtained to evaluate endothelium-dependent relaxation (Figure 5A). Endothelium-independent relaxation was assessed using a single dose of sodium nitroprusside (1 mM); (Figure 5B). HFD+F produced a significant reduction in the potency of acetylcholine. Similarly, the maximum response to acetylcholine was

![Figure 1: Changes in body composition and glucose sensitivity. Panel A represents the body weight at the beginning of the study (upper panel) and the body weight at the end of the study (lower panel). Epididymal fat collected at the end of the study is shown in panel C. Panel D represents the results of the glucose tolerance test. Data are mean±SEM of 5 animals.*P<0.05 and ***P<0.001, compared to the control group. HFD+F = high fat diet+fructose.](image-url)
Figure 2: Lipid Profile. Plasma levels of total cholesterol and triglycerides measured at the end of the study are shown in figure 2. Data are mean±SEM of 4-5 animals. *P<0.05, compared to the control group. HFD+F: high fat diet+fructose.

Figure 3: Renal Evaluation. Plasma levels of creatinine and urea (panel A and B, respectively). Morphology of the kidney of control (panel C) and high-fat diet+fructose (HFD+F; panel D). Glomeruli are shown by black arrows. Data are mean±SEM of 4-5 animals.

Discussion

In this study, we evaluated the effects of a high fat diet associated with ingestion of fructose on the function and morphology of the mesenteric artery, kidney and liver.

In human, obesity usually is a consequence of an imbalance between energy intake and expenditure, in favor of intake. However, in rats the models of obesity are often based in genetic models that are prone to obesity and sometimes high caloric or high fat diets fail to induce obesity and metabolic disorders. Some of the features of high fat diet resistant rats were described recently [8]. Similarly, variations in carbohydrate sensitivity are also reported, endorsing the difficult of studying rodent models of obesity [9]. In our study, twelve weeks of HFD+F increased the body weight gain, showing that the HFD+F was able to produce an experimental model of obesity based on a positive energy balance. Furthermore, after 12 weeks of diet and before sacrifice, animals were fasted for 14 hours and then subjected to a glucose tolerance test. We observed that fasting glucose was similar between groups, indicating that treatment with the HFD+F for 12 weeks induced obesity without significantly reduced in the mesenteric artery of the HFD+F group (Emax: 52 ± 12%) compared to the control group (Emax: 100 ± 13%).

The relaxation induced by the NO donor, sodium nitroprusside (SNP) was not changed in mesenteric artery of rats treated with high-fat diet+fructose (Figure 5B).

No morphological changes were observed in any of the three tunics of the mesenteric artery in the HFD+F group when compared to the control group. Tunica intima presented an intact endothelium, medium tunica without changes in the arrangement of smooth muscle cells and tunica adventitia with unchanged connective tissue (Figure 5C and 5D). After orcein staining both groups revealed the presence of elastic fibers intermingled with smooth muscle cells in the medium tunica, as well as internal and external elastic borders (data not shown).
the simultaneous onset of diabetes, which would be revealed by the high fasting glucose. After the dextrose overload, blood glucose was measured after 10, 20, 30, 40 and 60 minutes. The results show that only 60 minutes after overload, blood glucose was significantly higher in the HFD+F group, suggesting the beginning of insulin resistance after 12 weeks of treatment. It is in accordance to a previous study showing that 4 weeks of high fat diet induces insulin resistance euglycemic rats [11]. This is of particular interest for studying the effects of obesity, independent of the effects of diabetes.

In animal models of obesity, epididymal fat content has been correlated with abdominal fat in humans. As abdominal fat is a reference to the propensity for the development of cardiovascular diseases, increased epididymal fat content suggests that this pattern of diet might represent a high risk factor for cardiovascular disorders arising from obesity [11]. However, more studies are needed to confirm this hypothesis.

Although diets with high levels of carbohydrates or fats are often associated with elevation of plasma lipids, in experimental models of obesity, equally observed in human being, obesity is not necessarily accompanied by elevation of serum lipids [11]. Our data showed that rats treated with HFD+F had an increase in triglycerides levels with no change in total cholesterol concentration in plasma. Corroborating our data, a recent study showed that 18 weeks of high fat diet plus fructose induced insulin resistance in euglycemic rats [11]. Another study showed that a high fat diet altered cardiovascular parameters without changing the levels of total cholesterol, HDL and LDL fractions and triglycerides [13]. On the other hand, a recent study comparing different types of high fat diet for rodents, all of them made with lard, showed increased levels of fasting glucose, total cholesterol and triglycerides after 12 weeks of high fat diet consumption [14]. It suggests that the increase in plasma lipids depends on the type of lipid contained in the diet and individual metabolism, since the excess of carbohydrates will generate acetyl-CoA, which is the precursor of both triglycerides and cholesterol as well.

Obesity is also a risk for the development of renal and hepatic diseases. The risk of developing chronic kidney disease with obesity may occur due to the occurrence of other comorbidities such as diabetes mellitus and hypertension, and can be considered as an independent renal risk factor [15]. In our study, renal function was evaluated by plasma concentrations of creatinine and urea, which are metabolites filtered by the kidney, thus plasma accumulation represents poor renal filtration and therefore renal failure. We also evaluate the morphology of the kidneys. There was no morphological or functional changes in the kidneys of rats treated with high-fat diet plus fructose, suggesting that renal failure is probably a late event and secondary to other events not observed in this group of obese animals.

Additionally, we evaluated hepatic morphology and function of animals subjected to high-fat diet plus fructose. Data on literature reveals that obesity is an important risk factor for non-alcoholic fatty liver disease (steatosis) [16,17]. We evaluated seric levels of albumin and ALT as markers of hepatic dysfunction. There was no change in the levels of albumin and ALT in animals subjected to high-fat diet plus fructose. On the other hand, there was an increase in fat deposition observed by histological analysis of the obese rats’ liver. In human obese it was shown a decrease in albumin and increase in ALT levels in patients with non-alcoholic hepatic steatosis disease [16]. It is accepted that hepatic steatosis may occur in different levels of progression, and in the benign stage, hepatic steatosis is not accompanied by changes in biochemical markers, being called simple steatosis [18]. Our data suggest that the consumption of the HFD+F causes a simple hepatic steatosis, since hepatic function was not compromised, which probably might be a late event. We speculate that continuing on this diet, simple steatosis may progresses to non-alcoholic fatty liver disease.

In mesenteric artery, we evaluated the endothelium-dependent relaxation by concentration-response curves to acetylcholine (1 nM - 10 mM) while endothelium-independent relaxation was assessed.
by a single dose of sodium nitroprusside (1 mM). HFD+F produced a significant reduction in the potency and maximal response to acetylcholine in mesenteric artery. On the other hand, endothelium-independent relaxation produced by a single dose of sodium nitroprusside was not affected in HFD+F group. These data suggest that the HFD+F induced vascular dysfunction which was not related to damage or disturbances in the smooth muscle intracellular response since endothelium independent relaxation was not affected by the treatment and morphological changes were not seen as well. This finding confirms previous studies that showed reduced endothelium-dependent relaxation without changes in the endothelium-independent relaxation in another model of rat obesity and other models of risk factors for cardiovascular disease such as arterial hypertension and diabetes [19-22]. Together with the morphological analysis of the mesenteric artery showing that the intima and medium layers were preserved in HFD+F group, it is suggestive that the impaired endothelium-dependent relaxation in the HFD+F group was not associated with damage in the endothelial layer. However, it is possible associated to a reduction in nitric oxide bioavailability. More studies are needed to confirm this hypothesis.

In conclusion, our data show that the HFD+F induces obesity associated to an increase in adipose tissue accumulation as well as simple non-alcoholic hepatic steatosis. Structurally, the HFD+F did not affect the kidney and mesenteric artery. Functionally, the HFD+F decreased the endothelium-dependent relaxation (but not endothelium-independent) of the mesenteric artery, whereas kidney and liver presented no damages to their function.

Acknowledgement

Financial support: FAPESP and CNPq.

References

12. de Oliveira C1, Scarabelot VL2, Souza Ad3, Oliveira CM1, Medeiros LF2, et al. (2014) Obesity and chronic stress are able to desynchronize the temporal pattern of serum levels of leptin and triglycerides. Peptides 51: 46-53.


